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Enhancement of the *Brucella* AMOS PCR Assay for Differentiation of *Brucella abortus* Vaccine Strains S19 and RB51

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Because the brucellosis eradication program uses slaughter and quarantine as control measures, it would benefit from faster methods of bacterial identification. Distinguishing vaccine strains from strains that cause infections among vaccinated herds in the field is essential. To accomplish this, our PCR-based, species-specific assay (B. J. Bricker and S. M. Halling, J. Clin. Microbiol. 32:2660–2666, 1994) was updated to identify *Brucella abortus* vaccine strains S19 and RB51. Three new oligonucleotide primers were added to the five-primer multiplex *Brucella* AMOS PCR assay. Identification is based on the number and sizes of six products amplified by PCR.

Recently, we described a *Brucella* PCR assay which can distinguish *Brucella abortus* (biovars 1, 2, and 4), *Brucella melitensis* (biovars 1, 2, and 3), *Brucella ovis*, and *Brucella suis* (biovar 1) (1). The assay, herein designated "AMOS" (on the basis of the initials of the species that it identifies), recognizes all of the species and biovars of *Brucella* found in cattle in the United States. As described previously (1), the AMOS PCR assay is a multiplex primer assay that uses a five-primer cocktail. One primer anneals to the IS*711* element, while each of the other four primers hybridizes to one of the *Brucella* species at a locus nearby and at variable distances outside the element. As designed, *B. abortus* (biovars 1, 2, and 4) amplifies a 498-bp product, *B. melitensis* (all biovars) amplifies a 731-bp product, *B. ovis* amplifies a 976-bp product, and *B. suis* (biovar 1) amplifies a 285-bp product. One drawback of the AMOS assay is its inability to discriminate between the vaccine strains (S19 and RB51) and field strains from cattle with naturally occurring infections.

Strategy for identification of *B. abortus* RB51 by PCR. A rough mutant of *B. abortus* designated strain RB51 (8) is a possible vaccine candidate (2, 8). A major advantage of RB51 is that it does not appear to react in standard serological tests (2, 9, 10), thus decreasing the incidence of false-positive reactors. Studies on the efficacy of RB51 as a vaccine strain are encouraging (2, 8). We observed a polymorphism which appeared to be unique to RB51 and its parental strain, strain 2308. This polymorphism, which occurs at a locus containing the genetic element IS*711* (3), was chosen as the target site for a new oligonucleotide primer to further augment the AMOS PCR assay repertoire. This new primer was designed to differentiate *B. abortus* 2308 and RB51 from other *B. abortus* strains by PCR amplification of an additional 364-bp product.

Identification of a polymorphism in *B. abortus* RB51 and its parental strain, strain 2308. Genomic DNAs from selected strains of *B. abortus* biovar 1 were isolated and purified as described previously (4). The genomic DNAs (1.75 μg each) were digested with 5 U of the restriction enzyme *Cla*I according to the manufacturer's instructions. The restriction fragments (350 ng per lane) were resolved by agarose gel electrophoresis (0.8% SeaKem GTG agarose [FMC, Rockland, Maine] in 1× TBE [6]) and were blotted onto a nylon membrane (Hybond N; Amersham, Arlington Heights, Ill.). A digoxigenin-labelled IS*711* DNA probe was hybridized with bound fragments and was visualized according to the manufacturer's instructions (Genius Labelling Kit 3 no. 1175 041 and Luminescent Detection Kit 7 no. 1363 514; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). As shown previously (1), the IS*711* probe typically hybridizes to five or six *B. abortus* fragments including a fragment of 1,500 bp in size (see the arrow in Fig. 1). Neither 2308 nor RB51 hybridizes to a 1,500-bp fragment, but they both hybridize to a unique 2,000-bp fragment (Fig. 1). Subsequent DNA sequencing (dideoxy chain termination; Sequenase version 2.0; United States Biochemical, Cleveland, Ohio) of parent strain 2308 demonstrated two tandem copies of IS*711* at this locus. The DNA sequence showed that both copies were oriented in the same direction and were immediately adjacent to each other.

Selection of a new PCR primer specific for *B. abortus* 2308 and RB51 polymorphism. A new primer was formulated to function with the internal IS*711* PCR primer already contained in the AMOS PCR primer cocktail. This sixth oligonucleotide primer was computer designed (Oligo version 3.4 Primer Analysis Program; National Biosciences, Hamel, Minn.) to be compatible (nonannealing) with the other five primers in the cocktail and to amplify a product with an unique size that is easily differentiated from the typical *B. abortus* product. The new primer within the IS*711* sequence results in the amplification of a 364-bp product from the tandem copies of IS*711* in RB51 and 2308 and from any tandem copies of IS*711*, provided that the elements are oriented in the same direction. The sequences (5′ to 3′) of the new oligonucleotide primers for PCR are as follows: RB51/2308 primer, CCC-CGG-AAG-ATA-TGC-TTC-GAT-CC; *eri* primer 1, GCG-CCG-CGA-AGA-ACT-TAT-CAA; and *eri* primer 2, CGC-CAT-GTT-AGC-GGC-GGT-GA (see below for a discussion of the last two primers).

PCR amplification of DNA products from *B. abortus* strains. DNAs (10^6 killed bacteria or 5 ng of purified genomic DNA per 50 μl of assay mixture) from *B. abortus* 544, S19, 2308, and RB51 were cycled 35 times through a regimen of 1.2 min at 95°C, 2.0 min at 55.5°C, and 2.0 min at 72°C in the presence of the six-primer cocktail under the conditions described previously (1). The amplified products were separated in a 2.5%

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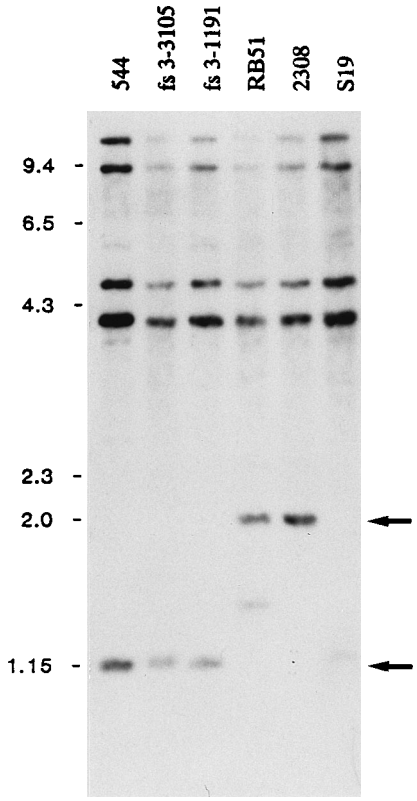

FIG. 1. Southern blot analysis. Genomic DNAs from six strains of *B. abortus* were digested with *Cla*I, resolved by electrophoresis, blotted onto a nylon membrane, and hybridized with digoxigenin-labelled IS*711* DNA as a probe. Numbers on the left reflect sizes (in kilobases). The sources of DNA are indicated above the lanes; fs indicates field isolates. Arrows mark the polymorphic change in one fragment from 1,150 to 2,000 bp.

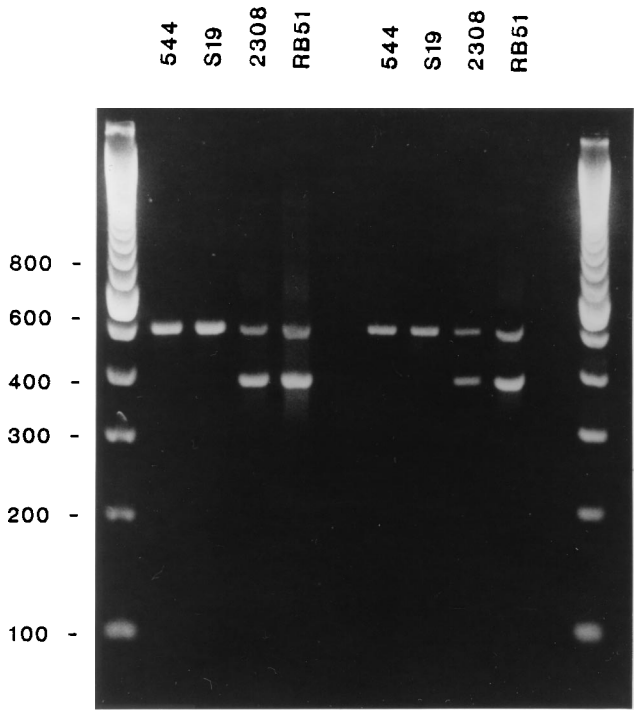

FIG. 2. PCR amplification products from *B. abortus* strains tested by the six-primer AMOS assay. PCR amplification was performed with four strains of bacteria (10^6 killed cells or 5 ng of purified genomic DNA) as described in the text. Five microliters of product was resolved by electrophoresis in agarose, visualized with ethidium bromide, and photographed. Numbers along the left margin indicate the sizes (in base pairs) of selected fragments of the 100-bp marker in lane 1. The sources of DNA are indicated above the lanes.

metaphor agarose gel (FMC) in the presence of 0.5× TBE and were visualized with ethidium bromide under UV light. The relative mobilities of the amplified products are shown in Fig. 2. As predicted, only one product (498 bp) was amplified from the DNAs of *B. abortus* 544 and S19; two products (498 and 364 bp) were amplified from 2308 and RB51 DNAs. The products are clearly differentiated by their electrophoretic mobilities in agarose gels.

Specificity of the 375-bp amplification product. We were uncertain how frequently adjoining copies of IS*711* arise in the *Brucella* genome. Previous studies suggest that the element is rarely mobile in most *Brucella* species and is stable once it has been inserted into the chromosome (4). We examined 100 field isolates of *B. abortus* randomly chosen from samples obtained from cattle throughout the United States (for identification of these field isolates, see reference 1). The isolates were tested with an abridged primer cocktail consisting of the *B. abortus*-specific primer, the IS*711* internal primer, and the new RB51 primer. All 100 isolates tested produced only the predicted 498-bp *B. abortus* product (data not shown). One of these isolates (NADC 1035) initially failed to produce an amplified product, but upon retesting the predicted product was amplified. The 364-bp product was amplified from only *B. abortus* 2308 and RB51, suggesting that adjacent copies of IS*711* are not commonly found in *B. abortus*. However, we did find that *B. ovis*, which has at least 30 copies of IS*711*, does appear to have a similar arrangement of tandem elements. As a result, *B. ovis* also amplifies a 364-bp product (data not shown). Because this product is so much smaller than the 976-bp product designed specifically for this species, we observed that the 364-bp product is sometimes preferentially amplified at the expense of the larger product. This does not confuse the identification of species, however, because *B. ovis* does not amplify the 498-bp product typical of *B. abortus* strains.

Strategy for the identification of *B. abortus* S19 by PCR. In the United States, cattle are only vaccinated with U.S. Department of Agriculture strain S19. Diagnostic laboratories are presented with *Brucella* isolates from vaccinated herds to determine if the isolate is the vaccine strain or a wild-type field isolate. While S19 can be distinguished from the wild type by culture methods, these methods are not rapid. A key element in the identification of strain S19 is its susceptibility to the addition of erythritol to the growth medium (5). This susceptibility is the result of a 702-bp deletion in a chromosomal region, designated the *eri* locus, which is associated with the ability to catabolize erythritol (7). Although Sangari et al. (7) described a PCR assay which can distinguish S19 from wild-type *B. abortus*, their assay is unable to identify the wild-type *Brucella* species. We wanted to incorporate the findings of Sangari et al. (7) into the AMOS PCR assay, but the sizes of their designated amplification products overlap the sizes of those used in the AMOS assay.

On the basis of their data, an additional oligonucleotide primer pair (see above) was computer designed (Oligo version 3.4 Primer Analysis Program). The first primer annealed to a region of the *eri* locus common to S19 and other *Brucella* species, while the second primer annealed within the 702-bp deletion.

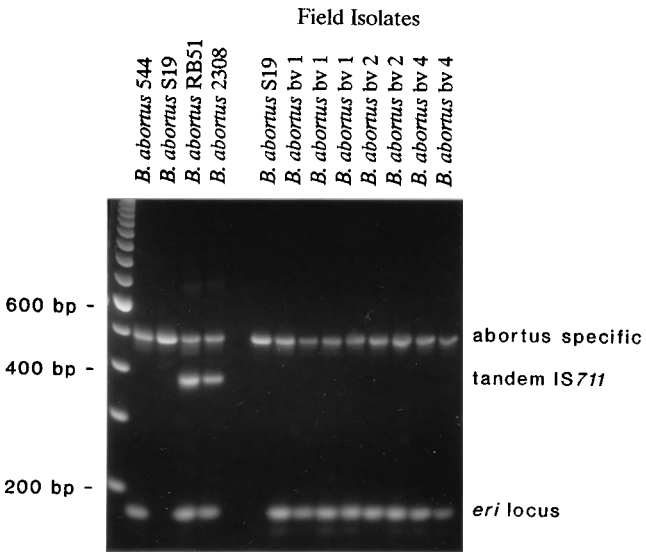

FIG. 3. PCR amplification products from the *B. abortus* strains tested by the eight-primer AMOS assay. PCR amplification was performed with 10^6 killed bacteria as described in the text. Five microliters of product was resolved by electrophoresis in agarose, visualized with ethidium bromide, and photographed. Lanes 2 to 4, amplification with four laboratory-adapted strains, including the two vaccine strains S19 and RB51; lanes 7 to 14, amplification of nine field isolates sent to the Diagnostic Bacteriology Laboratory (National Veterinary Services Laboratory) for identification, including one isolate identified as S19. Numbers along the left margin indicate the sizes (in base pairs) of selected fragments of the 100-bp marker in lane 1. The notations in the right margins indicate the amplified target. The sources of DNA are indicated above the lanes.

Identification of *B. abortus* S19 by PCR. DNAs (10^6 bacteria) from six *B. abortus* strains were assayed with the eight-primer cocktail (Fig. 3). All strains except S19 amplified a 178-bp fragment, indicating an intact *eri* sequence; strains 2308 and RB51 amplified a 364-bp product, indicating tandemly oriented IS*711* elements; and all six strains amplified the 498-bp product associated with *B. abortus*. Analysis of representative field isolates also showed agreement with the identifications made by the conventional methodology (Fig. 3). Analysis of 19 *Brucella* biovars by the eight-primer assay showed that, with the exception of S19, all 19 biovars (comprising six species) tested amplified the 178-bp product, indicating an intact *eri* sequence (data not shown).

The U.S. Department of Agriculture-derived vaccine used in the United States is consistently erythritol susceptible (5). Diagnostic laboratories occasionally receive field isolates that have all the characteristics of strain S19 except for susceptibility to the erythritol in the medium. Because the identification of strain S19 by PCR is based on the *eri* locus, we were particularly interested in how such samples behave in the AMOS assay. We obtained one erythritol-resistant S19 isolate (courtesy of Darla Ewalt at the Diagnostic Bacteriology Laboratory, Animal and Plant Health Inspection Service, Ames, Iowa) and found that it amplified only the 498-bp fragment, as is typical of strain S19 (data not shown).

The ability of the expanded AMOS assay to discriminate between *B. abortus* vaccine strains and wild-type isolates of *Brucella* enhances its value. We are in the process of transferring this technology to the Diagnostic Bacteriology Laboratory (National Veterinary Services Laboratory, Ames, Iowa) to see how well it functions in the clinical setting.

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